

A Possible Role for Chromium(III) in Genotoxicity

by Elizabeth T. Snow*

Chromium is found in the environment in two major forms: reduced Cr^{III} and Cr^{VI} , or chromate. Chromate, the most biologically active species, is readily taken up by living cells and reduced intracellularly, via reactive intermediates, to stable Cr^{III} species. Cr^{III} , the most abundant form of chromium in the environment, does not readily cross cell membranes and is relatively inactive *in vivo*. However, intracellular Cr^{III} can react slowly with both nucleic acids and proteins and can be genotoxic. We have investigated the genotoxicity of Cr^{III} *in vitro* using a DNA replication assay and *in vivo* by CaCl_2 -mediated transfection of chromium-treated DNA into *Escherichia coli*. When DNA replication was measured on a Cr^{III} -treated template using purified DNA polymerases (either bacterial or mammalian), both the rate of DNA replication and the amount of incorporation per polymerase binding event (processivity) were greatly increased relative to controls. When transfected into *E. coli*, Cr^{III} -treated M13mp2 bacteriophage DNA showed a dose-dependent increase in mutation frequency. These results suggest that Cr^{III} alters the interaction between the DNA template and the polymerase such that the binding strength of the DNA polymerase is increased and the fidelity of DNA replication is decreased. These interactions may contribute to the mutagenicity of chromium ions *in vivo* and suggest that Cr^{III} can contribute to chromium-mediated carcinogenesis.

Introduction

Chromate is a well-documented human and animal carcinogen. Occupational and environmental exposure to chromium has been widespread, and many studies of the biological effects of chromate compounds have been conducted (1-3). However, because of its complex intracellular metabolism, molecular mechanisms of chromium-induced genotoxicity are not well understood. Chromium genotoxicity and carcinogenesis are complex processes. Chromate ions are actively transported across cell membranes and are reduced intracellularly via reactive Cr^{V} and Cr^{IV} intermediates to stable Cr^{III} species. During this process, oxidative DNA damage and chromium-mediated crosslinks are produced, and point mutations are induced in a number of target genes. However, the mechanisms of chromium-induced mutagenesis are still unclear.

Numerous studies have attempted to determine which intracellular form of chromium or byproduct of chromium reduction is the ultimate carcinogenic and/or mutagenic species (3-5). Redox interactions resulting from chromium reduction (including oxidative base damage)

are generally considered the most likely cause of chromate mutagenesis. These processes have been implicated, in part, by increased chromate mutagenesis in A/T-specific Salmonella reversion strains (e.g., TA102) compared with that in G/C specific strains (TA100) (6); by the formation of 8-hydroxyguanine in chromium-treated DNA *in vitro* (7); and by the formation of active Cr^{V} species *in vitro* (3). Cr^{III} is also of interest because it can react with intracellular electrophiles, including nucleic acids, and is the most stable and plentiful form of chromium both in the environment and within the cell after chromate uptake and reduction. As much as 90% of the cellular chromium is present as the trivalent species (8). But Cr^{III} does not readily cross cell membranes and is relatively inactive *in vivo* and in cells in culture (9).

Experiments that have attempted to determine the carcinogenic and mutagenic potential of Cr^{III} compounds *in vivo* yielded negative or ambiguous results (2). Nevertheless, Cr^{III} compounds can reach the nucleus of cells within target organs (10) and, once inside the cell, Cr^{III} species can be mutagenic (11-13) and genotoxic (13). Recent evidence suggests that Cr^{III} species, although previously considered "kinetically inert," can readily bind to nucleic acids *in vitro* and can even act as redox agents when complexed to a conjugated aromatic ligand (11,14). Cr^{III} is also reported to induce a small (2-fold)

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increase in misincorporation by DNA polymerases during DNA replication *in vitro* (15,16). In addition, Cr^{III} ions bound to nuclear DNA increased RNA synthesis *in vitro* by increasing nonspecific initiation (17–19). This suggests that Cr^{III} might influence the regulation of gene transcription (17).

We have investigated the mechanisms of Cr^{III} genotoxicity *in vitro* using a DNA replication assay and *in vivo* by transfection of Cr^{III}-treated DNA into competent *E. coli* for scoring of survival and mutagenesis. Single-stranded M13 DNA was treated with low concentrations of CrCl₃ and excess chromium was removed by gel filtration. DNA replication was then measured on the treated templates. Both the rate of DNA replication and the amount of incorporation per polymerase binding event (processivity) were greatly increased on the chromium-treated template. This process is not polymerase dependent and has been seen with such diverse enzymes as *E. coli* DNA polymerase I and eukaryotic DNA polymerases α (20) and β . At an optimum concentration of 1 Cr^{III} ion bound per 300 nucleotides, the rate of synthesis by *E. coli* DNA polymerase I (Klenow) was increased 6-fold relative to the control. When transfected into *E. coli*, Cr^{III}-treated M13mp2 bacteriophage DNA showed a dose-dependent increase in mutation frequency up to 5-fold above background. These results suggest that Cr^{III} alters the structure of the DNA template resulting in both an increase in the binding strength of the DNA polymerase and a decrease in the fidelity of DNA replication. The combined effect of this template-polymerase association may be the unfaithful bypass of DNA lesions. The Cr^{III}-DNA interactions may thus contribute to both the mutagenicity of chromium ions *in vivo* and to chromium-mediated carcinogenesis by synergistically enhancing the mutagenicity of any DNA lesions produced during the intracellular reduction of Cr^{VI} *in vivo*.

Experimental Procedures

Materials

M13mp2 and M13mp7 single-stranded bacteriophage DNA was prepared using standard procedures (21). Unlabeled deoxynucleotides were obtained from Sigma Chemical Co. (St. Louis, MO); ³²P-labeled nucleotides and ⁵¹CrCl₃ (20.5 mCi/mmol) were from New England Nuclear (Boston, MA). The M13 17-mer sequencing primers were synthesized by Bernard Goldschmidt, Department of Environmental Medicine, New York University Medical Center. The T7 Sequenase kit was obtained from the United States Biochemical Corporation (Cleveland, OH). Polymerase I-Klenow fragment (pol I-KF) was obtained from Boehringer Mannheim (Indianapolis, IN). Cloned human DNA polymerase β (22) was a kind gift from Samuel Wilson of the National Cancer Institute. *Escherichia coli* JM101 was cultured from laboratory stocks maintained on minimal agar plates. Bacto-tryptone, agar, and yeast extract were

obtained from Difco laboratories (Detroit, MI). All other chemicals were of molecular biology or DNA grade, and all water was purified through a Milli-Q purification system. All CrCl₃ solutions were made fresh daily and diluted as required immediately before use.

Measurement of Cr^{III} Bound to M13mp2 DNA

M13mp2 single-stranded DNA was treated with ⁵¹CrCl₃ (0.4–50 μ M) at 37°C for 30 min in a 50- μ L reaction mixture containing 2 μ g of DNA, 0.2 mM Tris-HCl (pH 8.0), and 20 μ M EDTA. After incubation, the reaction mixtures were spun through Sephadex G-50 spin columns, prepared as described in Maniatis et al. (21), to remove the unbound ⁵¹Cr^{III} ions. The amount of ⁵¹Cr remaining bound to the DNA was determined by counting the samples in a NaI(Tl) scintillation detector coupled to a multichannel analyzer. ³H-TdR-labeled DNA was used in a control reaction to measure DNA recovery.

DNA Replication Using Cr-Treated M13mp2 Primer-Templates

M13 17-mer sequencing primer was 5' end-labeled with γ -³²P-ATP (3000 Ci/mmol) and T4 polynucleotide kinase (21). M13mp2 single-stranded DNA was hybridized with a 2-fold molar excess of either radiolabeled or cold primer in a 100- μ L reaction mixture containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 50 mM NaCl, and 10 mM dithiothreitol at 55°C for 10 min, then slowly cooled to room temperature. Chromium-treated templates were prepared by first reacting the DNA template with CrCl₃ for 30 min at 37°C and removing the excess chromium by gel filtration, as above. The treated DNA was then hybridized with 2-fold excess primer. Replication was carried out at 37°C for 15 min using 0.2 μ g of the modified DNA template and 0.04 units of pol I-KF in a 20- μ L reaction mixture containing 40 mM Tris-HCl (pH 7.4), 10 mM β -mercaptoethanol, 5 mM MgCl₂, and 50 μ M each dNTP (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate). The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and the amount of nucleotide incorporation was calculated from the 10% (w/v) trichloroacetic acid-precipitable counts collected on GF/C filters. Each determination was performed in triplicate and the results are presented as the average (\pm standard error).

Primer Extension Assays

Primer extension was carried out as above using M13mp7 DNA with a 5' end-labeled primer and 0.2 units of cloned DNA polymerase β in the presence of 0, 1, 5, or 10 μ M CrCl₃. (One unit of polymerase β equals 1 nmole deoxynucleotide monophosphate incorporated into activated calf thymus DNA in 30 min at 37°C.) The primed

DNA was mixed first with the CrCl_3 solution, the extension reactions were initiated by the addition of deoxynucleotide triphosphates and 5 mM MgCl_2 , incubated at 37°C for 15 min, and stopped by the addition of 0.5 M EDTA to yield a final concentration of 10 mM. Aliquots were transferred to 1.5-mL microtubes and mixed with loading buffer (99% deionized formamide, 10 mM Na_2EDTA , 10 mM NaOH , and 0.3% each xylene cyanol FF and bromphenol blue) then heated to 96°C for 3 to 4 min and rapidly chilled on ice before loading on an 8% (w/v) polyacrylamide-5 M urea gel which had been prerun for 15 to 30 min. Gel electrophoresis was carried out at 1600 to 1800 V until the dye front was within 2 in. of the bottom. The gel was then transferred to 3M Whatman filter paper and dried for 30 min at 70°C. The dried gel was placed with X-ray film (Kodak XAR-2) and exposed for 1 to 7 days at -70°C with an intensifying screen. Sequencing reactions were carried out using 5' end-labeled primers and templates, prepared as described above, and the "Sequenase" enzyme and buffers according to the manufacturer's instruction with the following modifications: 0.25 μM dATP was added to each of the termination buffers and the labeling mix was diluted 10-fold to increase production of short sequences.

Mutagenesis Assay

M13mp2 single-stranded DNA template (0.2 μg) primed with 15-mer was reacted with 0 to 10 μM CrCl_3 for 30 min at 37°C in a total volume of 50 μL in 50 mM Tris-HCl buffer (pH 7.4). The samples were then filtered through Sephadex G-50 spin columns (1000 rpm, 5 min) to remove free CrCl_3 . The metal-treated DNA (1–20 ng/sample) was used to transfect 0.2 mL of calcium-treated *E. coli* JM101 competent cells which were prepared using standard procedures (21). The average transfection efficiency of untreated, single-stranded M13mp2 DNA in JM101 was about 400 plaques per nanogram DNA. Aliquots of transfected cells (approximately 10 μL) were plated individually to give approximately 200 to 500 plaques/plate, as described previously (20). The plates were incubated overnight at 37°C, then nonmutant (dark blue) and colorless or light blue (mutant) plaques were counted after 16 to 24 hr at room temperature.

Results and Discussion

Both Cr^{III} and Cr^{VI} have been surprisingly refractory in producing mutagenic DNA damage *in vitro* (16,23). Although it is known that chromium produces significant DNA damage and mutagenesis *in vivo*, it is not clear what species of chromium ions produce mutagenic DNA damage or what specific type of mutagenic damage is produced by chromium. It has been postulated, for example, that Cr^{V} intermediates facilitate the production of oxidative damage within the cell and that Cr^{V} -glutathione conjugates may be involved in DNA-protein crosslinking [see Connett and Wetterhahn (24) for re-

view]. Cr^{III} , on the other hand, has been implicated as a DNA-DNA and DNA-protein crosslinking agent (25,26) but may also be mutagenic directly or by indirect interference with DNA replication fidelity and bypass of DNA lesions. Many different experimental approaches have been used to determine the mutagenic mechanism of Cr^{III} compounds. However, due to the poor ability of Cr^{III} ions to cross the cell membrane *in vivo*, these studies have generally proven negative. In contrast, DNA replication *in vitro* using synthetic polynucleotides and/or ϕX174 DNA templates in the presence of CrCl_3 or CrCl_2 (which rapidly becomes Cr^{III} in aqueous solution) shows a chromium-dependent increase in the frequency of nucleotide misincorporation (15,16,23). Experiments by Tkeshelashvili et al. (16), for example, have shown that *in vitro* incubation of $\phi\text{X174am3}$ DNA with CrO_3 produces a 2-fold increase in reversion frequency.

In our preliminary experiments (20), the effects of CrCl_3 on DNA replication *in vitro* were examined in the presence of both template-bound Cr^{III} and free Cr^{III} ions. Under these conditions, low concentrations of Cr^{III} increased, then decreased, DNA polymerase activity and also promoted increased polymerase processivity (20). In subsequent experiments the inhibition of DNA replication by free Cr^{III} ions (21,29) was eliminated by removal of unbound Cr^{III} ions before DNA replication was begun. Chromium binding to DNA is a relatively slow process (data not shown), and a preincubation time of 30 min was chosen for convenience. Cr^{III} binding to DNA under these conditions is dose dependent, as shown in Figure 1. This binding is primarily electrostatic in nature, since 40% or more the bound ions can be displaced by a 15-min high salt wash (0.5 M NaCl) at 37°C, and only 10 to 20% of the chromium is chelatable by 20 mM EDTA (a kinetically slow process) under the same conditions (re-

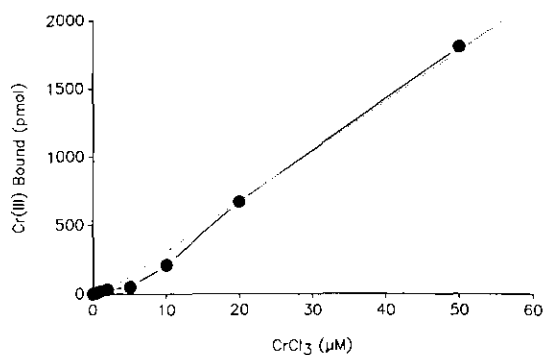


FIGURE 1. Cr^{III} binding to DNA *in vitro*. A total of 2 μg single-stranded M13mp2 DNA per reaction was incubated with $^{51}\text{CrCl}_3$ in a total volume of 50 μL TE/50 (0.2 mM Tris-HCl, pH 8, 20 μM EDTA) at 37°C for 30 min. Nonbound chromium was then removed by gel filtration through a 1-mL Sephadex G-50 spin column at room temperature. The amount of chromium remaining bound to the DNA template after G-50 chromatography (●) was determined by using a NaI scintillation counter. DNA recovery determined in a separate experiment using ^3H -labeled M13 DNA was found to average $85 \pm 3\%$.

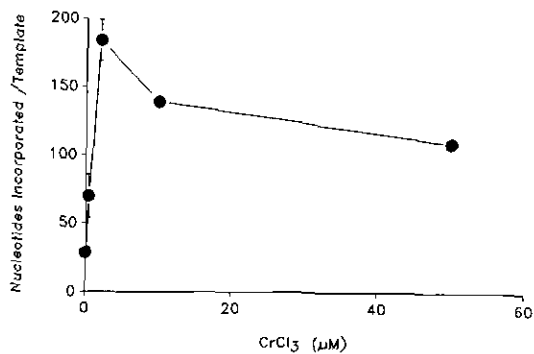


FIGURE 2. Nucleotide incorporation on a chromium-bound template and binding of CrCl_3 to single-stranded M13mp2 DNA. Single-stranded M13 DNA was treated for 30 min at 37°C with 0 to $50\ \mu\text{M}$ CrCl_3 in a total volume of $50\ \mu\text{L}$, and the unbound chromium was removed by gel exclusion chromatography, as described in the legend to Figure 1. DNA replication was then carried out on $0.2\ \mu\text{g}$ of treated template, and the trichloroacetic acid precipitable nucleotide incorporation was measured (●).

sults not shown). It is probable that this initial binding of relatively low concentrations of CrCl_3 is to the phosphate backbone of the DNA (see discussion below).

Both nucleotide incorporation and primer extension were then examined using the chromium-treated template. Figure 2 shows the results of nucleotide incorporation in which DNA templates pretreated with as little as $0.4\ \mu\text{M}$ CrCl_3 exhibited 2-fold increased nucleotide incorporation, while pretreatment with $2\ \mu\text{M}$ CrCl_3 produced a 6.3-fold increase in nucleotide incorporation. The amount of Cr^{III} bound to the template under the conditions used for these experiments was determined by measuring the amount of radioactive ^{51}Cr bound to the DNA after gel filtration through Sephadex G-50 (Fig. 1). As shown here, the rate of nucleotide incorporation is greatest on a template treated with $2\ \mu\text{M}$ CrCl_3 . At this concentration the treated template contains approximately 1 bound Cr^{III} ion per 300 nucleotides (25 Cr^{III} ions/template). However, even DNA templates treated with $50\ \mu\text{M}$ CrCl_3 which contain as many as one chromium ion bound for every six nucleotides, still show over a 4-fold increase in nucleotide incorporation relative to the untreated control.

We also studied nucleotide incorporation by primer-extension analysis using a $5'$ - ^{32}P -labeled oligonucleotide-primed M13mp2 template to determine the length of the extended primers on sequencing gels, as shown in Figure 3. This assay allows us to investigate sequence-specific polymerase pausing and termination with the same template and primer used in the incorporation assays. Our previous results suggested that polymerase processivity can be increased by low concentrations of chromium and that either DNA-DNA or DNA-protein crosslinking may also be increased at doses of 5 to $10\ \mu\text{M}$ Cr^{III} (20). These results were further confirmed by sequencing gel analysis of replication on chromium-treated templates in the absence of free chromium. Af-

FIGURE 3. Cr^{III} increases primer extension by cloned eukaryotic DNA polymerase β on a single-stranded template. M13mp7 was primed with a $5'$ -end-labeled sequencing primer, as described in "Experimental Procedures" and replicated with DNA polymerase β in the presence of CrCl_3 at 37°C for 15 min. The products were analyzed on an 8% sequencing gel adjacent to ddTTP and ddGTP sequencing reactions prepared using the T7 Sequenase kit with the same template and end-labeled primer in the absence of chromium.

ter treatment of the template with 1 to $5\ \mu\text{M}$ CrCl_3 , the extended primers are much longer than those seen in the absence of chromium, and the increased length is dose dependent. These results suggest that it is the Cr^{III} ions that are bound to the DNA template, but not the free chromium, which produces increased poly-

merase processivity. The results shown here pertain to DNA polymerase β , but similar results have been seen with DNA polymerase α (20) and *E. coli* polymerase I (20; Snow et al., unpublished observations).

In our previous study, when M13mp2 double-stranded DNA was treated with CrCl_3 (0.5 mM) and transfected into *E. coli*, the mutation frequency increased only 2-fold above the control (20). More positive results have since been obtained with M13mp2 single-stranded phage. This mutagenesis assay, in contrast to the ϕ X174 reversion assay, can detect a wide variety of mutations within the regulatory and coding sequences of the *lacZ α* gene. When primed single-stranded, M13mp2 DNA templates were pretreated with 1 to 10 μM CrCl_3 and separated from unbound Cr^{III} ions by Sephadex G-50 gel filtration prior to transfection into competent JM101 cells, the mutation frequency is increased in a dose-dependent manner up to 5-fold above background (Table 1). The concurrent decrease in phage recovery is indicative of CrCl_3 toxicity at concentrations as low as 1 μM . Consistent with reports from other laboratories (2), we have also observed that the CrCl_3 can also induce dose-dependent DNA-DNA crosslinking (20). Such crosslinks are expected to stop DNA replication (28,29) and may correlate with the toxicity of CrCl_3 in the mutagenesis assay with *E. coli*. The toxicity of Cr^{III} in the present study was similar to that reported by Schaaper et al. (23). Since DNA-protein crosslinking is also mediated by Cr^{III} *in vitro* (26) it might be involved in the inhibition of DNA replication observed at higher concentrations of chromium (20).

These observations of Cr^{III} -mediated increases in polymerase processivity and mutagenesis are very similar to the effects seen with low (micromolar) concentrations of manganese (MnCl_2). Magnesium ions are usually required as cofactors for DNA polymerase activity. However, MnCl_2 has been shown to substitute for MgCl_2 during DNA replication *in vitro* (30). At low concentrations, manganese binds preferentially to the phosphate backbone of the DNA template (31,32) and, like chromium, both increases DNA polymerase processivity (33) and decreases fidelity (32,34). Manganese has been shown to be mutagenic *in vitro* (8,15) and *in vivo* (35) and can increase polymerase-mediated nucleotide insertion opposite DNA lesions (36). Manganese

also increases the production of nontargeted base substitutions and frameshift mutations (37) and nonspecific initiation of RNA transcription (38). Cr^{III} has likewise been shown to increase nonspecific initiation by RNA polymerase *in vitro* (17-19) and *in vivo* (18) and may act by a similar mechanism.

The increased binding of DNA polymerases to DNA templates by Cr^{III} or Mn^{II} may specifically increase bypass across DNA-damage or noncoding lesions. Polymerase infidelity during DNA replication past the oxidative damage sites potentially produced during the intracellular reduction of chromium could partially account for the high mutagenic and carcinogenic potential of chromate *in vivo*. A model for Cr^{III} -mediated mutagenesis is shown in Figure 4. Under normal conditions, a replicative DNA polymerase encounters a DNA lesion, pauses, and allows time for DNA repair enzymes to recognize and remove the lesion. The polymerase then continues accurate replication of the template. In the presence of small amounts of Cr^{III} , however, the polymerase is more tightly bound to the template, and either may not stop at a DNA lesion or may pause too briefly to allow proper recognition of the lesion by DNA repair enzymes. The polymerase may also bind more tightly to the nucleotide precursors, which could promote direct misincorporation. DNA polymerase I in the presence of manganese, for example exhibits lower K_m values for both correct and incorrect nucleotide precursors and shows decreased selectivity for the correct base pair (39,40; E. T. Snow, unpublished observations). An increased probability that polymerase will insert an incorrect base opposite a DNA lesion will facilitate by-

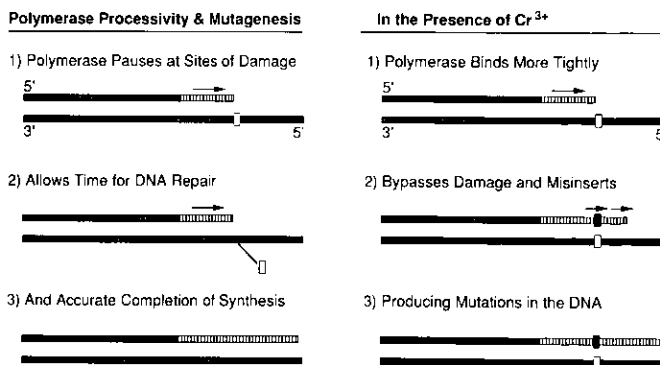


FIGURE 4. A model for Cr^{III} -mediated mutagenesis by polymerase bypass. As shown on the left, 1) a replicative DNA polymerase is expected to pause at sites of DNA damage, which will 2) allow time for DNA repair enzymes to recognize and remove the DNA lesion and 3) promote the subsequent accurate completion of DNA replication. However, in the presence of Cr^{III} , as shown on the right, 1) the DNA polymerase is more tightly bound to the template, making it more processive and also less faithful. Under these circumstances, when the polymerase encounters a lesion, it is less likely to pause or fall off the template and does not allow recognition of the lesion. Instead, 2) the polymerase is more likely to bypass the lesion and insert an incorrect base opposite the damaged or miscoding base. This could result 3) in the production of a mutation opposite the lesion and, consequently, could also lead to increased tumorigenesis.

Table 1. Mutagenesis of CrCl_3 -treated M13 DNA in *E. coli*.^a

CrCl_3 , μM	Survival, %	Mutant plaques/ total plaques	Mutation frequency, $\times 10^{-5}$
0	100	2/22,495	9
1	82.4	16/48,928	33
5	37.8	10/22,403	45
10	18.1	5/10,796	46

^aMutagenesis of the *lacZ* gene in M13mp2 was determined after transfection of chromium-treated phage DNA into *E. coli* JM101, as described in "Experimental Procedures." Survival was measured as the total number of plaques per nanogram of transfected DNA relative to the untreated control (400 plaques/ng).

pass of the damage site, producing a mutation in the process. Alternatively, Cr^{III} , also like manganese (31), is known to bind preferentially to guanine-containing DNA (41,42). This may result in direct mispairing or may promote oxidation (?) or depurination (23) of the guanine, causing subsequent mispairing of the modified (or missing) base.

The exact mechanisms of Cr^{III} mutagenesis and Cr^{III} -mediated crosslinking or production of oxidative damage *in vivo* are still far from clear; however, the effects of Cr^{III} , which are shown here to occur at extremely low concentrations, could be significant at the low doses of chromium that are characteristic of environmental exposures to chromate or after limited cellular uptake of environmentally plentiful Cr^{III} .

I thank Li-Sha Xu for her expert technical assistance and Samuel Wilson, who generously provided the cloned DNA polymerase β . The research described in this paper was supported by a National Cancer Institute grant (CA 45664) and a National Institute of Environmental Health Sciences Center grant (ES 00260).

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